The Plant Cell, Vol. 7, 1655–1666, October 1995

C-Terminal Deletion Analysis of Plant Plasma Membrane H^+-ATPase: Yeast as a Model System for Solute Transport across the Plant Plasma Membrane

Birgitte Regenberg,[a] José Manuel Villalba,[b] Frank C. Lanfermeijer,[a] and Michael G. Palmgren[a,1]

[a] Department of Plant Biology, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Copenhagen, Denmark

[b] Departamento de Biologia Celular, Universidad de Cordoba, Avenida San Alberto Magno, s/n, E-14004 Cordoba, Spain

The plasma membrane proton pump (H^+-ATPase) energizes solute uptake by secondary transporters. Wild-type Arabidopsis plasma membrane H^+-ATPase (AHA2) and truncated H^+-ATPases lacking 38, 51, 61, 66, 77, 92, 96, and 104 C-terminal amino acids were produced in yeast. All AHA2 species were correctly targeted to the yeast plasma membrane and, in addition, accumulated in internal membranes. Removal of 38 C-terminal residues from AHA2 produced a high-affinity state of plant H^+-ATPase with a low K_m value (0.1 mM) for ATP. Removal of an additional 12 amino acids from the C terminus resulted in a significant increase in molecular activity of the enzyme. There was a close correlation between molecular activity of the various plant H^+-ATPase species and their ability to complement mutants of the endogenous yeast plasma membrane H^+-ATPase (*pma1*). This correlation demonstrates that, at least in this heterologous host, activation of H^+-ATPase is a prerequisite for proper energization of the plasma membrane.

INTRODUCTION

In both plant and yeast cells, transport across the plasma membrane is driven by an electrochemical gradient of protons. Therefore, both types of cells have primary transport systems that generate proton gradients (that is, plasma membrane H^+-ATPases) and secondary transport systems that utilize those gradients. Thus, in terms of chemiosmotic circuits at the plasma membrane, the yeast *Saccharomyces cerevisiae* constitutes a good model for the plant cell.

The molecular cloning of secondary transport systems in the plant plasma membrane by functional complementation in yeast has appeared as a powerful new tool in plant transport biology. The following secondary transport systems of the plant plasma membrane have been cloned by complementation of yeast mutants defective in the respective uptake systems: (1) two K^+ channels (Anderson et al., 1992; Sentenac et al., 1992), (2) a K^+/H^+ cotransporter (Schachtman and Schroeder, 1994), (3) several amino acid transport systems (Frommer et al., 1993, 1994; Hsu et al., 1993; Kwart et al., 1993), (4) an NH_4^+ transporter (Ninnemann et al., 1994), (5) a peptide transporter (Steiner et al., 1994), (6) a sucrose transporter (Riesmeier et al., 1992), and (7) three sulfate transporters (Smith et al., 1995). In addition, (8) a plant glucose transporter (Sauer et al., 1990) and (9) two sucrose transporters (Sauer and Stolz, 1994), cloned by heterologous probing, are able to complement yeast transport mutants.

The Arabidopsis plasma membrane H^+-ATPase gene family has been cloned using classical cloning strategies (Harper et al., 1989, 1990, 1994; Pardo and Serrano, 1989a, 1989b; Houlné and Boutry, 1994). Three isoforms of the Arabidopsis plasma membrane H^+-ATPase (AHA1, AHA2, and AHA3) have been expressed and studied in yeast (Villalba et al., 1992; Palmgren and Christensen, 1993, 1994). The unexpected result in these experiments was that a mutation in the yeast H^+-ATPase could not be complemented in situ by a plant H^+-ATPase gene. Actually, functional plant H^+-ATPases are indeed expressed to high levels in yeast, but most of the enzyme is retained in the endoplasmic reticulum (ER; Villalba et al., 1992). Thus, the lack of complementation was suggested as being due to a targeting default specific for plant H^+-ATPase within its heterologous host, the yeast cell (Villalba et al., 1992).

The C terminus of H^+-ATPase serves as a negative regulator of H^+-ATPase activity (Serrano et al., 1992). The recent success in overexpressing the plasma membrane H^+-ATPase in yeast (Villalba et al., 1992) has facilitated a genetic approach to the study of this C-terminal domain. The first mutant made using this system was an H^+-ATPase deprived of 92 amino acids at the C terminus (Palmgren and Christensen, 1993). The truncated enzyme is found mainly in the yeast plasma membrane and supports normal yeast growth. It was therefore suggested that the C terminus of the plant H^+-ATPase hinders exit of the enzyme from the yeast ER or passage of the H^+-ATPase through the secretory pathway (Palmgren and Christensen, 1993).

In our study, we examined the cellular localization of AHA2 by immunolocalization of the plant H^+-ATPase in yeast-derived

[1] To whom correspondence should be addressed. Current address: Department of Molecular Biology, Copenhagen University, Øster Farimagsgade 2A, DK-1353 Copenhagen K, Denmark.

spheroplasts. Wild-type AHA2 clearly could be localized to the plasma membrane in addition to high levels of enzyme being retained in the ER. The ability of the plant enzyme to support yeast growth in the absence of the endogenous yeast plasma membrane H^+-ATPase protein (PMA1) was concomitant with an increase in the molecular activity (at least fivefold) because amino acids were removed from the C terminus of AHA2. This suggests that the lack of complementation by wild-type AHA2 is due to the low molecular activity of this enzyme and not related to a default in targeting within the cell. Therefore, at least in the yeast model system, activation of H^+-ATPase is essential for proper energization of the plasma membrane.

RESULTS

Expression of Wild-Type and Truncated Plant H^+-ATPase in Yeast

As a first step in the structural and functional analysis of the C-terminal portion of plant plasma membrane H^+-ATPase, a series of eight genes was synthesized that encode sequential C-terminal deletions of AHA2. The genes were generated by introducing stop codons through site-directed mutagenesis in the 3′ end of the coding region using the mutagenic oligonucleotides depicted in Table 1. The corresponding truncated H^+-ATPases were designated aha2Δ38, aha2Δ51, aha2Δ61, aha2Δ66, aha2Δ77, aha2Δ92, aha2Δ96, and aha2Δ104, according to the number of amino acids deleted from the C-terminal end of the AHA2 polypeptide. The primary structure of the C-terminal end of each protein is shown in Figure 1. Some properties of aha2Δ92 have already been described (Palmgren and Christensen, 1993). All eight coding regions were placed under the transcriptional control of the yeast promoter *PMA1*. The *PMA1* promoter confers high levels of constitutive expression. There is, however, a small degree of regulation according to carbon source, with glucose boosting expression two- to threefold (Villalba et al., 1992). The resulting chimeric genes were subcloned into a high-copy-number yeast plasmid and introduced into yeast.

Immunoblot analysis with a polyclonal antibody specific for the central part of plant H^+-ATPase (amino acids 340 to 650 of AHA3; Palmgren et al., 1991) revealed that the transformed yeast lines accumulated truncated H^+-ATPase proteins in the ER for each deletion (Figure 2A). The changes in electrophoretic mobility of the truncated plant H^+-ATPases confirmed the deletions at the protein level. As shown in Figure 2A, each of the mutants was produced at a somewhat lower level compared to the level attained for wild-type AHA2. The transformed yeast cells, however, expressed each truncated H^+-ATPase to sufficient levels for a detailed analysis as given below.

Plant H^+-ATPase Accumulates in Internal Membranes but Is Also Correctly Targeted to the Yeast Plasma Membrane

Plant H^+-ATPase expressed in yeast accumulates in internal membranes (Palmgren and Christensen, 1993). Accordingly, AHA2 and the truncated forms of the enzyme were present in the ER of transformed yeast cells grown on glucose medium and constituted a significant fraction of the ER membrane protein as determined by the intensity of Coomassie Brilliant Blue R 250 staining of ER membrane proteins separated by SDS-PAGE (Figure 3A). Immunoreactive plant H^+-ATPase was quantified following protein gel blotting. A dilution series of recombinant AHA2 protein, tagged with six histidine residues at the N terminus between Asp-6 and Ile-7 and purified by

Table 1. Nomenclature of Plasmids, Transformed Yeast Strains, ATPase Allele Expressed, and Sequences of Mutagenic Oligonucleotides

Plasmid	Yeast Strain	ATPase Allele Expressed	Mutagenic Oligonucleotide[a]	Reference
	RS72			Cid et al. (1987)
pMP136	MP142	AHA2		Palmgren and Christensen (1993)
pMP303	MP375	aha2Δ38	GCTGAGCAAGCTTAGAGAAGAGCTGAG	This study
pMP342	MP374	aha2Δ51	GAGAAATGAAGTTATCGCGAATTGTCTGAG	This study
pMP135	MP198	aha2Δ61	CACGGTTTACAGCCTTAAGAAGCTGTTAAC	This study
pMP341	MP381	aha2Δ66	GCTCAAAGGACCCTTTAGGGTTTACAGCC	This study
pMP210	MP380	aha2Δ77	AGAGAGATAGGCTCCATGGGCA	This study
pMP132	MP194	aha2Δ92	TGAGAACTAGACGTCTTTCAC	Palmgren and Christensen (1993)
pMP285	MP378	aha2Δ96	GGCTCAACTAGTTTGAGAA	This study
pMP284	MP377	aha2Δ104	GATACATCTAGAGCGGAAA	This study
pRS891	MP383	PMA1		Villalba et al. (1992)
YEp351	RS933			Villalba et al. (1992)

[a] Underlining indicates changes. Orientation is given in 5′ → 3′.

```
          * * *  ** *** * * **        *** *   *   **  ** * ******              **   *  ********* ** ********** * *****     **
AHA2      TYFPLDVFKFAIRYILSGKAWLNLFENKTAFTMKKDYGKEERFAQWALAQRTLHGLQPKEAVNIFPEKGSYRELSEIAEQAKRRAEIARLRELHTLKGHVESVVKLKGLDIETPSHYTV   948
aha2Δ38   TYFPLDVFKFAIRYILSGKAWLNLFENKTAFTMKKDYGKEERFAQWALAQRTLHGLQPKEAVNIFPEKGSYRELSEIAEQA    910
aha2Δ51   TYFPLDVFKFAIRYILSGKAWLNLFENKTAFTMKKDYGKEERFAQWALAQRTLHGLQPKEAVNIFPEK   897
aha2Δ61   TYFPLDVFKFAIRYILSGKAWLNLFENKTAFTMKKDYGKEERFAQWALAQRTLHGLQP   887
aha2Δ66   TYFPLDVFKFAIRYILSGKAWLNLFENKTAFTMKKDYGKEERFAQWALAQRTL   882
aha2Δ77   TYFPLDVFKFAIRYILSGKAWLNLFENKTAFTMKKDYGKEER   871
aha2Δ92   TYFPLDVFKFAIRYILSGKAWLNLFEN   856
aha2Δ96   TYFPLDVFKFAIRYILSGKAWLN   852
aha2Δ104  TYFPLDVFKFAIRYI   844
```

Figure 1. C-Terminal Sequences Encoded by Deletion Mutants of Arabidopsis *AHA2*.

The hydrophilic C-terminal amino acid stretch following the last putative membrane spanning segment (underlined) is shown. Stop codons were introduced into the reading frame of *AHA2* by virtue of site-directed mutagenesis. The primary structure of the corresponding truncated proteins is indicated. The number at the end of each line indicates the position of the last C-terminal amino acid. Amino acids conserved in 11 cloned plant plasma membrane H+-ATPases (see alignment in Perez and Boutry, 1994) are represented by asterisks.

Ni^{2+}–nitrilotriacetic acid chromatography (F.C. Lanfermeijer and M.G. Palmgren, unpublished data; Figure 3C), was used as a standard for quantification (Figure 2B). Depending on the construct, immunoreactive plant H+-ATPase constituted 2 to 29% of the ER protein (Table 2).

Although a major fraction of immunoreactive AHA2 polypeptide was found to accumulate in membranes of probable ER origin, expression in the plasma membrane also seemed to be a possibility. Thus, when total membranes were subjected to sucrose density gradient centrifugation, a fraction of the AHA2 polypeptide comigrated with yeast plasma membrane H+-ATPase. Plasma membranes from glucose-grown yeast expressing AHA2 were purified by two subsequent sucrose density gradient centrifugation steps (Figure 3B). In this way, contamination by the substantial amount of AHA2 diffusing from the major ER band in sucrose gradients (Palmgren and Christensen, 1993) was avoided. The immunoreactive plant H+-ATPase polypeptide in the yeast plasma membrane (Figure 2B) amounted to 3 to 13% of the plasma membrane protein (Table 2). The levels of AHA2 (13%) and aha2Δ38 (11%) tended to be somewhat higher than those of the other constructs.

Immunolocalization experiments with yeast cells (Figure 4), using a monoclonal antibody specific for plant H+-ATPase, confirmed that the various plant plasma membrane H+-ATPase species were correctly targeted to the plasma membrane of yeast. Although a substantial part of AHA2 was located in membranes surrounding the nucleus and in secretory vesicles, AHA2 showed distinct peripheral staining, an indication of plasma membrane localization. Perinuclear staining was somewhat heterogeneous, suggesting that the expression level in the ER varied considerably between individual yeast cells. Plasma membrane staining, however, was always evident. H+-ATPases lacking 38 and 51 amino acids showed the same intracellular distribution as wild-type AHA2, the expression pattern of aha2Δ61 was somewhat intermediate, and the ATPases devoid of >61 amino acids were all predominantly expressed in the plasma membrane. Immunolocalization was performed on yeast cells grown on galactose medium, that is, when expression of plant H+-ATPase was low (Palmgren and Christensen, 1993). When immunolocalization was performed on yeast cells cultured on glucose medium (to boost production of plant H+-ATPase), plant H+-ATPase was also detected in the plasma membrane (data not shown). In addition, we observed a substantial accumulation of all constructs in internal membranes.

C-Terminal Deletion Analysis of Plant H+-ATPase Reveals Domains for Regulation of ATP Affinity and Molecular Activity

To test the hypothesis that domains in the C-terminal end of the plant H+-ATPase serve a regulatory function, we

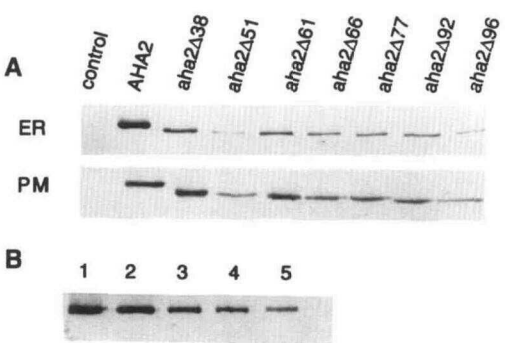

Figure 2. Immunodetection of Plant H+-ATPase in ER and Plasma Membranes from Yeast Strains Expressing C-Terminally Truncated AHA2.

The primary antibody used was a polyclonal antibody raised against the central portion of AHA3 (amino acids 340 to 650). Protein gel blots were subsequently incubated with a ^{125}I-labeled anti–rabbit antibody, and immunodecorated bands were visualized using a PhosphorImager.
(A) Shown is the protein gel blot of the ER and plasma membranes (PM; 5 μg of protein per lane in 8% acrylamide gels).
(B) Protein gel blot of AHA2 containing an N-terminal 6×histidine tag. The recombinant protein was affinity purified by Ni^{2+}–nitrilotriacetic acid chromatography and used as a standard for immunoquantification. Lane 1 contains 2 μg of protein; lane 2, 1 μg; lane 3, 0.5 μg; lane 4, 0.25 μg; and lane 5, 0.125 μg.

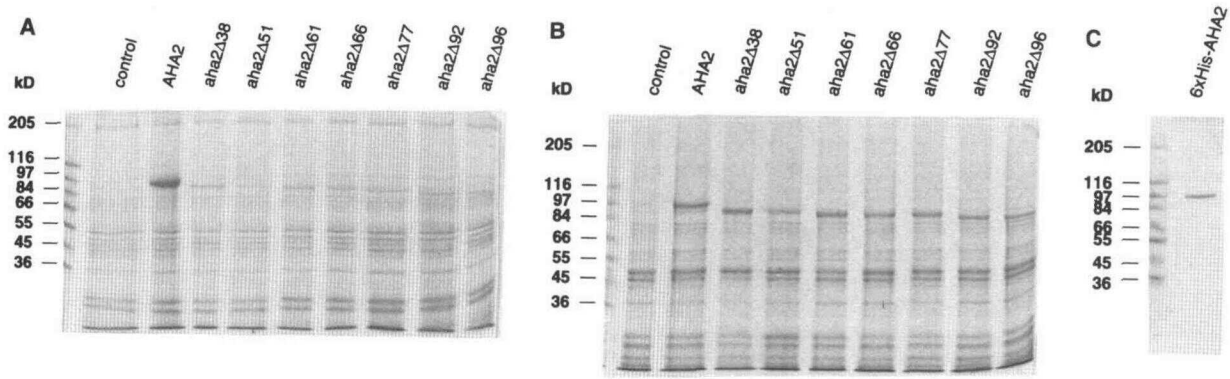

Figure 3. Polypeptide Composition of ER and Plasma Membranes from Yeast Strains Expressing C-Terminally Truncated AHA2.

Yeast was transferred from galactose to glucose medium and grown either for 4 hr (for the isolation of ER) or to saturation for 24 hr (for the isolation of plasma membranes) before being harvested. Yeast ATPase was expressed only in galactose medium (in the plasma membrane), whereas plant ATPases were expressed in both galactose and glucose medium. Some residual yeast H^+-ATPase (20 to 25%), however, remains in the plasma membrane. Membrane protein (20 μg per lane) from each strain was subjected to SDS-PAGE, and polypeptides were visualized by staining gels with Coomassie blue. The position of plant H^+-ATPase is seen as a novel band around the position of the 97-kD molecular weight marker.

(A) Polypeptide composition of ER.

(B) Polypeptide composition of plasma membranes.

(C) Protein gel of histidine-tagged AHA2 (6xHis-AHA2; 1 μg) affinity purified by Ni^{2+}-nitrilotriacetic acid chromatography.

investigated the kinetic properties of each of the truncated plant enzymes produced in the yeast.

pH Optimum

Each of the plant H^+-ATPase species expressed in the ER of transformed yeast cells was an active ATPase. ER vesicles containing wild-type AHA2 matched the pH dependence typically observed with plasma membrane H^+-ATPase isolated from plant sources (Figure 5A). The pH optimum was 6.5, and activity dropped rapidly at higher values. On the other hand, the pH optimum of all mutated proteins was shifted toward neutral pH (pH 7.0; Figure 5A).

K_m *for ATP and* V_{max}

The apparent affinity for ATP of the various truncated proteins from the yeast ER was measured with an ATP regenerating system. With 10 mM Mg^{2+} and ATP levels from 47 to 4000 μM, all mutated proteins obeyed Michaelis-Menten kinetics (Figure 5B). When ATP dependence was measured at pH 7.0, wild-type plant H^+-ATPase exhibited a relatively high K_m (2.0 mM) value, whereas all mutants exhibited a low K_m (0.1 mM) value.

The results of a kinetic analysis of the V_{max} and K_m values for the hydrolysis of ATP as a function of pH are shown in Figure 6. The V_{max} of AHA2 has a pH optimum at 6.7 (Figure 6A), but all the truncated proteins had a pH optimum displaced to more alkaline values (pH 7.2 to 7.4; exemplified by aha2Δ77 in Figure 6A). Between pH 6.5 and 7.0, K_m of AHA2 increased severalfold (from 50 μM to ~2 mM ATP; Figure 6B). However, the deletion mutants (as exemplified by aha2Δ77; Figure 6B) displayed a high affinity for ATP (K_m between 50 and 500 μM) in the pH range from 6.2 to 8.0.

Vanadate Sensitivity

Vanadate is a potent inhibitor of enzymes that form phosphorylated intermediates during their reaction cycle (Cantley et al., 1978). All plant H^+-ATPases were strongly inhibited by vanadate (as exemplified by AHA2, aha2Δ51, aha2Δ66, and aha2Δ77; Figure 5C). All of the deletion mutants were characterized as having a two- to threefold higher sensitivity for vanadate as compared with AHA2 (apparent K_i values for vanadate are ~10 μM for AHA2 and ~4 μM for all truncated H^+-ATPases; see Figure 5C).

Effect of Lysophosphatidylcholine

Lysophosphatidylcholine is an activator of plant plasma membrane H^+-ATPase (Palmgren, 1991). When the ATPase activity of the ER vesicles was titrated with this lipid, AHA2 was activated approximately two- to fourfold as previously reported (Palmgren and Christensen, 1994), whereas the ATPase activities of the truncated proteins were unaffected by the addition of up to 65 μg/mL of lysophosphatidylcholine (Figure 5D).

Specific Activity

The increase in molecular activity was difficult to quantify accurately because the expression level varied considerably between the different species of aha2. Thus, the differences in specific ATPase activity between plant H^+-ATPases

expressed as outlined in Table 2 could reflect either differences in the amount of the various enzymes expressed or fundamentally different rates of turnover of the catalytic cycle of the different enzymes. To address this issue, the amount of immunoreactive plant H+-ATPase polypeptide in ER and plasma membranes was quantified after protein gel blot transfer of the protein to nitrocellulose (Figure 2 and Table 2) and correlated with the specific ATPase activity of ER and plasma membranes, respectively. This allowed us to determine the molecular activity of the different aha2 constructs (Table 2).

Removal of 38 amino acids had only a minor effect on specific molecular activity (Table 2). However, compared with AHA2, the truncated ATPase that was devoid of 51 or more C-terminal residues had a five- to 10-fold higher turnover rate for ATP hydrolysis. The molecular activities of the constructs in the ER were approximately twofold higher than those of the plasma membranes (Table 2). However, plasma membrane vesicles isolated from yeast are of mixed sidedness (Monk et al., 1989). Accordingly, the ATPase activity of the plasma membranes increased approximately twofold when Triton X-100 was added (data not shown). Triton X-100 unmasks the latent ATP binding sites. It had no effect on the ATPase activity of ER vesicles.

Table 2. ATP Hydrolytic Activity and H+-ATPase Content of ER and Plasma Membranes[a]

ATPase Allele Expressed	Specific ATPase Activity[b]		H+-ATPase Content (%)[c]		Molecular ATPase Activity[d]	
	ER	PM	ER	PM	ER	PM
AHA2	0.33	0.11	29	13	1.1	0.9
aha2Δ38	0.14	0.07	11	11	1.3	0.7
aha2Δ51	0.16	0.20	2	3	8.5	5.8
aha2Δ61	0.74	0.48	8	8	9.2	5.9
aha2Δ66	0.36	0.37	4	6	9.3	6.1
aha2Δ77	0.32	0.31	4	6	9.0	5.0
aha2Δ92	0.50	0.35	7	7	7.4	5.1
aha2Δ96	0.18	0.23	3	3	6.6	7.0
aha2Δ104	0.18	0.32	3	5	5.7	6.8
None[e]	0.04	0.02	—	—	—	—

[a] H+-ATPase was quantified after protein blot transfer, as described in Methods. Data are the mean of four experiments (SD within 10%) with the same membrane preparation. Similar values (within 20%) for molecular activities were obtained with two different purified membrane preparations isolated independently.

[b] Micromoles of Pi released per minute per milligram of membrane protein (pH 7.0 at 24°C). PM, plasma membrane.

[c] H+-ATPase polypeptide relative to total protein (%).

[d] Micromoles of Pi released per minute per milligram H+-ATPase polypeptide (pH 7.0 at 24°C).

[e] Transformed with YEp351 (empty plasmid).

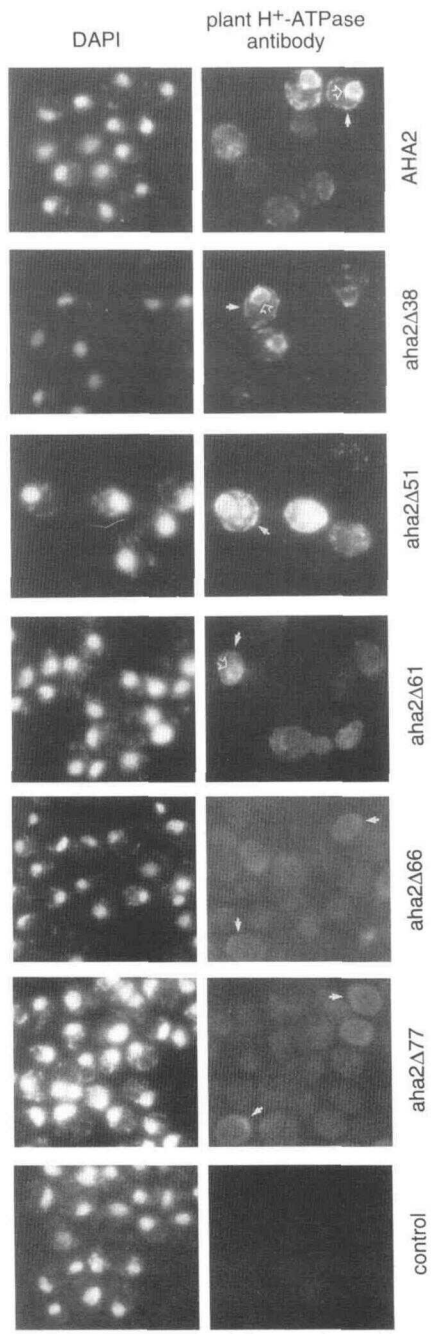

Figure 4. Immunofluorescense Labeling of Yeast Spheroplasts Producing C-Terminally Truncated AHA2.

At left are 4',6-diamidino-2-phenylindole (DAPI)–stained nuclei; at right are fluorescein 5-isothiocyanate second antibody conjugates in the same cells. A monoclonal antibody raised against plant H+-ATPase was used. Open arrowheads point at staining surrounding the nucleus, indicative of ER localization in yeast. Filled white arrowheads show peripheral staining, indicative of plasma membrane localization. Yeast cells were grown on galactose medium. Magnification is ×1100.

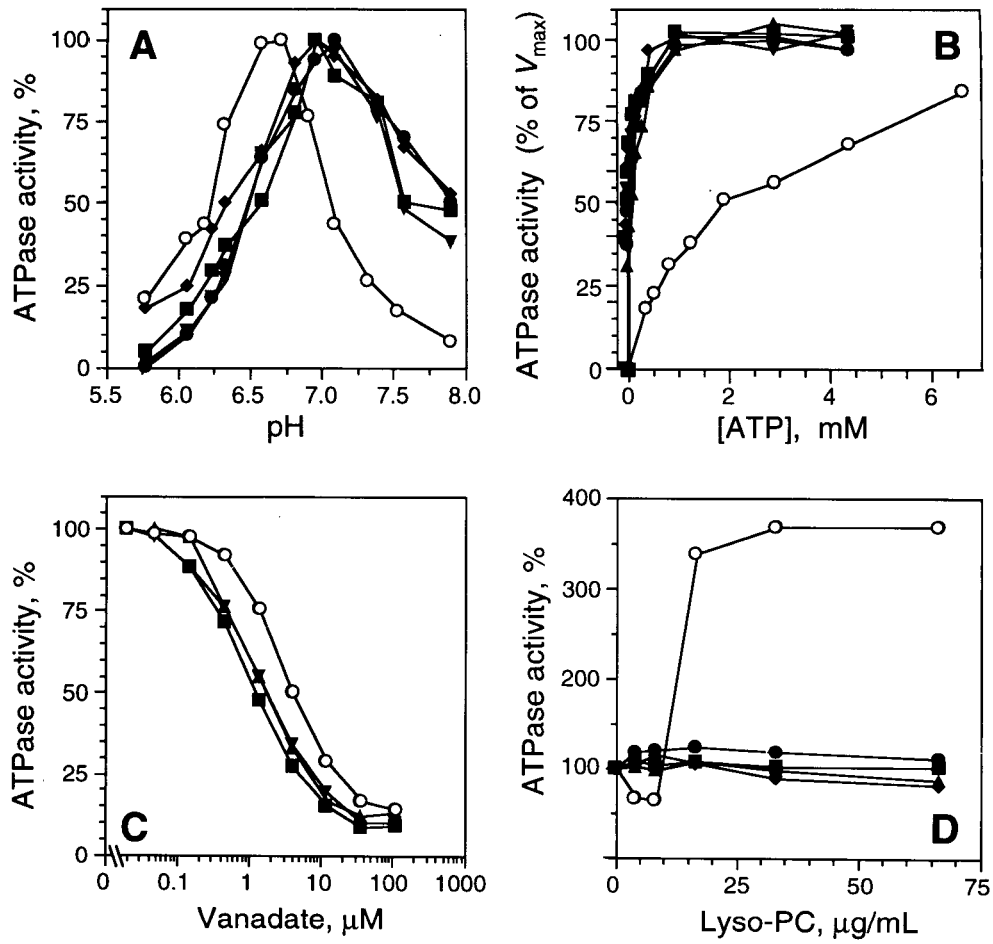

Figure 5. Kinetic Properties of C-Terminally Truncated AHA2 Produced in Yeast.

Purified ER membranes were prepared by sucrose gradient centrifugation from transformed yeast strains grown on glucose medium.
(A) pH dependence measured with 2 mM ATP. The H^+-ATPase activities (100%) at optimal pH are as follows (units are given in micromoles of Pi released per milligram of protein per minute): AHA2, 0.88; aha2Δ38, 0.12; aha2Δ51, 0.24; aha2Δ66, 0.28; and aha2Δ77, 0.28. The specific ATPase activity of ER vesicles isolated from yeast cells transformed with an empty expression plasmid (strain RS933) was 0.03 to 0.09 μmol of Pi released per milligram of protein per minute, depending on pH (optimum towards alkaline pH). ○, AHA2; ♦, aha2Δ38; ▼, aha2Δ51; ■, aha2Δ66; ●, aha2Δ77.
(B) Effect of ATP concentration on ATPase activity at pH 7.0.
(C) Sensitivity to vanadate measured at pH 7.0 and with 2 mM ATP.
(D) Effect of lysophosphatidylcholine (Lyso-PC) measured at pH 7.0 and with 2 mM ATP.

Activity State of Plant H^+-ATPase Determines Its Ability To Complement *pma1*

In the yeast strain RS72 (Cid et al., 1987) used in our studies, the natural promoter of the yeast H^+-ATPase has been replaced by a galactose-dependent (*GAL1*) promoter. Expression of plasmid-borne plant H^+-ATPase is under the control of the constitutive *PMA1* promoter (see above). Thus, when the yeast cells are maintained on galactose medium, both the yeast and the plant H^+-ATPases are expressed. After transfer to glucose medium, only plant H^+-ATPase is produced, and to grow, yeast cells become dependent on the plant enzyme.

In this study, yeast cells expressing wild-type AHA2 but no endogenous yeast ATPase were barely able to grow on glucose medium (Figure 7; Palmgren and Christensen, 1993). Control cells transformed with an empty expression plasmid did not grow at all (Figure 7).

Removal of 38 C-terminal amino acids from the plant ATPase had no positive effect on the growth rate of transformed cells in the absence of PMA1 (pH in the growth medium was 5.7;

Figures 7 and 8). Removal of 66 to 104 residues produced an optimal growth rate of transformed cells that approximated the growth rate of cells expressing plasmid-borne *PMA1*. The growth rates in liquid minimal medium increased more or less gradually when 38 to 66 amino acids were removed (Figure 8). There seemed to be a positive correlation between the increase in V_{max} and the ability to support yeast growth in the absence of PMA1 (Figure 8). Although differences in the amount of plant H+-ATPase are evident in the plasma membrane (Table 2), they cannot explain the growth properties of transformed yeast cells. Thus, it tends to be the least active constructs (AHA2 and aha2Δ38) that have the highest expression level in both the ER and plasma membrane (Table 2). Complementation of *pma1* by the truncated plant H+-ATPase was still partial, however, because cells expressing plant H+-ATPase deprived of 66 to 104 residues grew significantly slower on acidic medium (pH 3.0) than yeast cells producing PMA1 (data not shown).

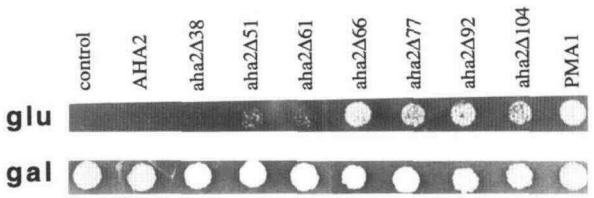

Figure 7. Drop Test for the Growth of Yeast Strains Expressing C-Terminally Truncated AHA2.

Approximately 10^3 cells in 5 μL of water were spotted on plates containing either 2% galactose (gal) or 2% glucose (glu). Growth was recorded after 3 days (glucose plates) or 4 days (galactose plates). Yeast ATPase was expressed alone in galactose medium, whereas plant ATPases were also expressed in glucose medium. The control strain had been transformed with an empty expression plasmid. PMA1 indicates yeast strain transformed with the expression plasmid harboring the *PMA1* gene.

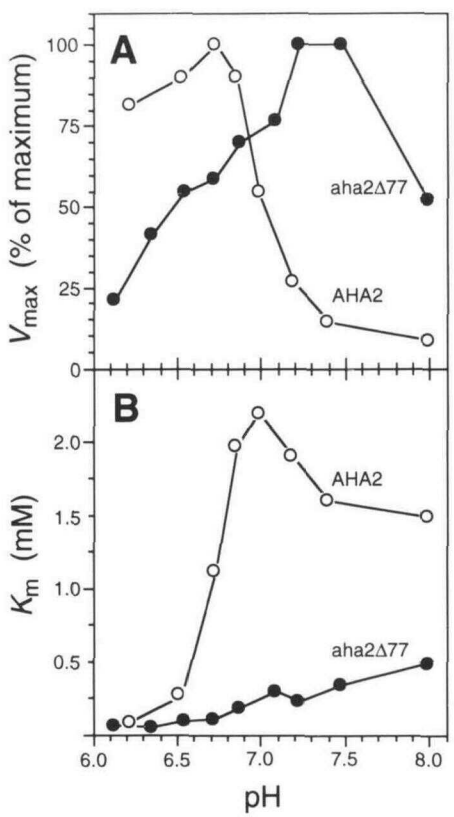

Figure 6. V_{max} and K_m of AHA2 and ahaΔ77 as a Function of pH.

Purified ER membranes containing either AHA2 (○) or aha2Δ77 (●) were prepared by sucrose gradient centrifugation from transformed yeast cells grown on glucose medium.

(A) V_{max} for ATP as a function of pH. The V_{max} values (100%) at optimum pH for each preparation used in this experiment are as follows (units are given in micromoles of Pi released per milligram of protein per minute): AHA2, 0.95; aha2Δ77, 0.11.

(B) K_m for ATP as a function of pH.

DISCUSSION

Correct Targeting of the Plant H+-ATPase Expressed in Yeast

We found that all AHA2 species were correctly targeted to the yeast plasma membrane (Figures 2 to 4). Therefore, mistargeting in yeast does not explain why plant H+-ATPase does not complement *pma1*. Transport of integral membrane proteins to the yeast vacuole seems to occur by default, suggesting that yeast plasma membrane proteins contain positive sorting information necessary for their localization (Nothwehr and Stevens, 1994). Because plant plasma membrane proteins, including AHA2, seem to be correctly targeted when expressed in yeast, this sorting information must be shared between yeast and plant plasma membrane proteins. Despite the very high expression level of AHA2 in the ER, the relative amount of this protein in the plasma membrane was reduced compared with the other constructs (Figures 2 and 3, and Table 2). The maximum capacity of the yeast plasma membrane to accommodate H+-ATPase is probably determined by the rate at which H+-ATPase enters the secretory pathway. Due to the very high expression level of AHA2, it is possible that this polypeptide saturates the secretory pathway and subsequently accumulates in the ER in a nonspecific manner (Villalba et al., 1992).

This hypothesis is supported by the observation that the expression level influences the intracellular distribution of plant H+-ATPase. Thus, in individual yeast cells expressing reduced amounts of plant H+-ATPase, expression in the plasma membrane is always evident, even when there is no accumulation

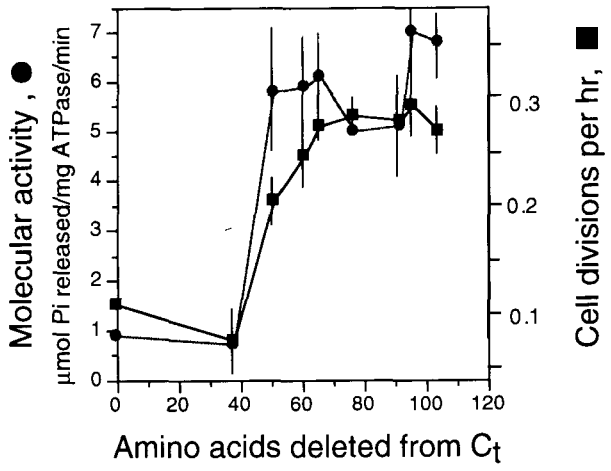

Figure 8. Correlation between Growth Rate of Yeast Strains Expressing C-Terminally Truncated AHA2 and Molecular Activity of the Protein in Yeast Plasma Membranes.

Growth (■) was recorded by measuring changes in the $A_{660\ nm}$ of suspensions of yeast cells in liquid minimal medium containing glucose (2%). On this medium, the yeast cells did not express endogenous plasma membrane H^+-ATPase. Data for molecular activities (●) are the mean of at least three independent membrane isolations for each mutant. Standard deviation is indicated by vertical lines. C_t, C terminus.

of protein in internal membranes. In addition, when the general level of expression is low, for example, when yeast cells are grown on galactose medium, truncated H^+-ATPase is barely detectable in internal membranes (Figure 4), whereas on glucose medium, where expression is high (Villalba et al., 1992), accumulation in the ER is evident (Figures 2A and 3A). The accumulation of protein in the ER has been observed previously when heterologous proteins (Wright et al., 1988) and even homologous proteins (Supply et al., 1993) are expressed at high levels in yeast and other organisms; this suggests that ER accumulation is the consequence of an overload effect.

Regulation of ATP Affinity and Molecular Activity

Removal of 38 C-terminal residues from AHA2 produced a high-affinity state of the enzyme. This state was characterized by a shift in the pH optimum of the enzyme from pH 6.5 to 7.0 (at 3 mM Mg-ATP; Figure 5A), and a decreased K_m for ATP (approximately fivefold at pH 7.0; Figure 5B). The increase in ATP affinity was most pronounced at a neutral pH and was neglible below pH 6.0 (Figure 6). Deleting >38 amino acids to a total of 104 amino acids did not result in additional changes in the kinetics of the H^+-ATPase. Whereas the pH sensitivity of an enzyme is most often an indication of an ionizable group at the site of the enzyme reaction, it can also be a measure of some change in the tertiary structure of the enzyme that affects the active site. The data presented here suggest that protonation of an ionizable group in the AHA2 polypeptide with a pK of ~6.7 is sufficient to induce the high-affinity state of the enzyme. The pK at 6.7 could be identified with the imidazole group of histidine that would be unprotonated in the low-affinity state of the enzyme. Because the pH dependence of ATP affinity is lost when the 38 C-terminal amino acids were truncated, the group that titrated at approximately pH 6.7 might be located within this stretch of amino acids. Two conserved histidine residues (His-923 and His-928) are located in the extreme C-terminal end of the AHA2 polypeptide and might be involved in the pH-dependent activation of AHA2. Alternatively, the group protonated at ~6.7 pH could be located elsewhere in the polypeptide, for example, close to or within the ATP binding site, where protonation of the residue could be important for binding the C-terminal autoinhibitory amino acids to the active site.

The phytotoxin fusicoccin, produced by the fungus *Fusicoccum amygdali*, stimulates proton extrusion from all higher plant tissues tested thus far. Fusicoccin increases the rate of seed germination, elongation growth, and nutrient uptake as well as causing stomata to stay open. These phenomena are all thought to be coupled to proton extrusion (for a review, see Marrè, 1979). Treatment of intact plant tissues with fusicoccin induces an activated state of the plasma membrane H^+-ATPase with altered kinetics compared with the native enzyme. The activated enzyme exhibits a more alkaline pH optimum and often a higher affinity for ATP than the nonactivated enzyme. The fusicoccin-activated enzyme cannot be activated further by tryptic removal of the hydrophilic C terminus of the enzyme (Johansson et al., 1993; Rasi-Caldogno et al., 1993; Lanfermeijer and Prins, 1994). Our data support the hypothesis that the activation of H^+-ATPase by fusicoccin is mediated by the C-terminal regulatory domain.

We have previously detected phospholipase A activity in plant plasma membranes and suggested that a product of this enzyme, lysophosphatidylcholine, is a natural regulator of plasma membrane H^+-ATPase activity (Palmgren et al., 1988). Interestingly, the deletion mutants of AHA2 devoid of 38 or more C-terminal residues are insensitive to this lipid activator (Figure 5D). This suggests that the lysophosphatidylcholine binding site is already lost in aha2Δ38. The amino acid residues in the extreme C-terminal end are very hydrophilic (42% charged residues) and would constitute an unusual binding site for lipids. Alternatively, lysophosphatidylcholine binds to a different portion of the polypeptide and induces a conformational change that allows the plant H^+-ATPase to switch from the low-affinity state to the high-affinity state already adopted by aha2Δ38.

In this analysis, we showed that a five- to 10-fold increase in molecular activity is apparent when 51 or more residues are deleted, whereas removal of 38 amino acids was not sufficient to increase molecular activity (Table 2). However, in these kinds of experiments, there is no way to check for the amount of active versus inactive or misfolded enzyme. An approach

for future studies would be to determine the amounts of accessible, correctly folded ATP-binding sites by, for example, affinity labeling with radiolabeled 5′-(*p*-fluorosulfonyl)benzoyladenosine (Brauer and Tu, 1995).

Complementation of *pma1* by *AHA2*

There seems to be a correlation between the molecular activity of plant H+-ATPase (Table 2), growth rate (Figure 8), and the amount of enzyme accumulating in internal membranes of yeast cells grown on galactose medium when endogenous yeast H+-ATPase is also produced (Figure 4). Thus, *AHA2* was expressed at very high levels, whereas the mutated genes encoding hyperactive ATPases depleted of more than 61 residues showed greatly reduced expression in internal membranes (Figure 3 and Table 2). We suggest that production of hyperactive ATPase can be detrimental for the yeast cell because it will consume a significant proportion of ATP present in the cells. Selection is therefore likely to be against yeast cells producing surplus amounts of these constructs.

As residues are removed from the C terminus of AHA2, there is a more or less gradual increase in the ability of the enzyme to support yeast growth in the absence of PMA1. A maximum was obtained in the H+-ATPase lacking 66 amino acids. Differential expression of plant H+-ATPases in the yeast plasma membrane is not likely to account for the very different growth phenotypes of yeast expressing these enzymes. Therefore, it seemed reasonable that differences in intrinsic properties of these enzymes could explain their differential ability in substituting for PMA1. The V_{max} was the only kinetic parameter of AHA2 found to be correlated with the growth phenotype of transformed yeast cells (Figure 8). Therefore, we suggest that this increase in molecular activity determines the ability of *AHA2* to complement *pma1*.

Yeast H+-ATPase itself cannot support growth on glucose medium unless the enzyme is activated (Eraso and Portillo, 1994). Perhaps plant H+-ATPases cannot support yeast growth on glucose medium because they are never activated in this heterologous organism. An explanation might be that plant H+-ATPase does not serve as a substrate for the regulatory protein kinase(s) of yeast (Eraso and Portillo, 1994) that activates the yeast H+-ATPase in response to glucose.

The plant plasma membrane is energized in vivo by plant growth factors, such as auxin and blue light, and phytotoxins, such as fusicoccin. Whether this energization is due to post-translational activation of plasma membrane H+-ATPase (Palmgren, 1991; Johansson et al., 1993), an increase in the amount of H+-ATPase in the plasma membrane (Hager et al., 1991), or modification of secondary transport systems (Blatt, 1988) has been much discussed. The data presented in this paper demonstrate that activation of the H+-ATPase protein is sufficient to have a dramatic impact on the growth of yeast cells, depending on this heterologous H+-ATPase. By analogy, we suggest that, in the plant cell, post-translational activation of H+-ATPase is an important determinant for establishing the steep electrochemical H+ gradients required for energization of nutrient uptake in the roots, phloem loading and/or unloading, opening of stomata, and elongation growth.

Moreover, it now seems possible to replace not only yeast secondary transporters but also primary transporters by their plant counterparts. This opens new avenues for using yeast as a model system to study solute transport across the plant plasma membrane. A first task would be to study the coupling between the activity state of plant H+-ATPase and solute uptake through secondary plant transporters. In the near future, it may be possible to transfer elements of signal transduction pathways, which are responsible for regulating a plant transporter, into yeast, thus opening the way to functional dissection of regulatory pathways.

METHODS

Strains, Media, and Microbiological Techniques

Saccharomyces cerevisiae RS72 (Cid et al., 1987) and its transformants were used in this study. RS72 (*MATa ade1-100 his4-519 leu2-3,112*) carries the yeast plasma membrane H+-ATPase *PMA1* gene under control of the galactose-dependent galactokinase (*GAL1*) promoter. Cells were grown in medium with 2% glucose or galactose plus 0.7% yeast nitrogen base (without amino acids; Difco) supplemented with adenine (40 μg/mL) and L-histidine (30 μg/mL). The medium was buffered with 50 mM succinic acid adjusted to pH 5.7, or pH 3.0 when indicated, with Tris. Solid media contained 2% agar.

Yeast transformation was performed according to the standard protocol of Ito et al. (1983). After transformation, the cells were plated on galactose medium without leucine, and the transformants were selected after 4 days of growth at 30°C.

Construction of Truncated Mutants of H+-ATPase

Plasmid pMP136 containing the AHA2 (isoform 2 of *Arabidopsis thaliana* plasma membrane H+-ATPase) cDNA for expression in yeast has been described previously (Palmgren and Christensen, 1993). This plasmid is a derivative of YEp-351 (Hill et al., 1986) and contains the AHA2 cDNA in the correct orientation between the strong promoter and termination regions of the yeast plasma membrane H+-ATPase (*PMA1*) gene, allowing for efficient expression in yeast (Villalba et al., 1992). In addition, the plasmid contains the *LEU2* gene, encoding β-isopropylmalate dehydrogenase, for selection and a 2-micron circle for high-copy-number replication.

Site-directed mutagenesis was performed on a 1.0-kb KpnI-XbaI fragment of the gene encoding the 3′ translated region of the gene subcloned in pBluescript SK− (Stratagene). New stop codons were introduced by virtue of the overlap extension technique (Ho et al., 1989) in which polymerase chain reaction is used. In addition, restriction sites were introduced in the sequence either encompassing or preceding the stop codon. This made identification of the mutant genes in plasmid minipreparations possible. The fusion product of the polymerase chain reaction was subcloned in pBluescript SK−. At this point, the insert was sequenced in its entirety by the dideoxy chain termination

procedure (Sanger et al., 1977) to ensure that no unexpected mutations had occurred.

The mutated KpnI-XbaI fragment was excised and substituted with the corresponding fragment in the yeast expression plasmid pMP136 (see above). Subcloning was performed in a three-in-one ligation step in which the 1.0-kb KpnI-XbaI fragment containing the desired mutation(s) in the 3′ end of *AHA2*, a 2.0-kb XhoI-KpnI fragment of pMP136 containing the 5′ end of the *AHA2* gene, and a 7.7-kb XhoI-SpeI fragment of pMP136 containing the promoter and terminator regions of *PMA1* as well as the YEp351 sequence were fused.

Homogenization and Membrane Preparation

Yeast plasma membranes were purified by differential and sucrose gradient centrifugation (Villalba et al., 1992). Microsomal membranes were resuspended in 1 mL of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 20% [w/w] sucrose (buffer STED 20), layered on top of a 12-mL sucrose step gradient (2.5 mL each of 50, 42, 33, and 29% [w/w] sucrose in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and centrifuged for 16 hr at 30,000 rpm (TST 41.14 rotor; Kontron Instruments, Milan, Italy). Endoplasmic reticulum (ER) membranes were collected from the 29/33% interphase, and plasma membranes were collected from the 42/50% interface. After twofold dilution in STED 20, the membranes were centrifuged at 50,000 rpm (70 Ti rotor; Beckman Instruments International S.A., Geneva, Switzerland) for 45 min, and the pellet was resuspended in STED 20 supplied with 1 mM phenylmethylsulfonyl fluoride and 0.1 mg/mL chymostatin. The membrane fractions were frozen in liquid nitrogen and stored at −80°C.

ATPase Assay and Protein Determination

ATPase activity was assayed according to a modified protocol of Baginsky et al. (1967) with 1 to 3 μg of membrane protein at 30°C. The assay medium (300 μL) contained 20 mM 2-(*N*-morpholino)ethanesulfonic acid, 20 mM 3-(*N*-morpholino)propanesulfonic acid, 50 mM KNO_3, 5 mM NaN_3, 3.5 mM sodium molybdate, 10 mM $MgCl_2$, and the indicated concentrations of ATP. The pH was adjusted to the desired value with *N*-methyl-D-glucamine. After 20 min, the reaction was stopped by the addition of 300 μL of ice-cold stop solution made by mixing 10 mL of 102 mM ascorbic acid, 0.3 N HCl, and 0.065% SDS with 1 mL of 57 mM NH_4-heptamolybdate. The tubes were incubated for 10 min on ice to allow for formation of the Pi–molybdate complex. Excess molybdate was complexed by the addition of 450 μL of a solution containing 154 mM $NaAsO_2$, 68 mM Na_3-citrate, and 350 mM acetic acid. After 60 min at room temperature, the color stabilized, and absorbance at 860 nm was determined.

ATP hydrolysis was also measured in a spectrophotometric assay by coupling ATP hydrolysis to NADH oxidation (Palmgren, 1991). In this case, 0.3 mM NADH, 2.4 mM phospho*enol*pyruvate (neutralized with KOH), 33 μg of pyruvate kinase, and 33 μg of lactate dehydrogenase were included in the assay medium.

Protein concentration was determined by the method of Bradford (1976), with the Bio-Rad protein assay reagent and bovine gamma globulin as the standard.

Gel Electrophoresis and Protein Gel Blotting

ER and plasma membrane proteins were separated by SDS-PAGE on 8 or 10% acrylamide using the system of Laemmli (1970). Protein gel blotting was essentially as described by Villalba et al. (1992). After electrotransfer of polypeptides to an Immobilon-P (Millipore) membrane, the membrane was immunolabeled with a polyclonal antibody (No. 721; diluted 4000 times) specific for the central part of plant H^+-ATPase (amino acids 340 to 650 of AHA3; Palmgren et al., 1991), followed by incubation of the membrane with 0.56 μg (7.5 μCi) of ^{125}I-labeled anti-rabbit antibody (Amersham International) in a total volume of 25 mL of blocking buffer. After careful washing of the membrane, the amount of bound radioactivity was quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). AHA2 containing an N-terminal 6×histidine tag between Asp-6 and Ile-7 was affinity purified by Ni^{2+}-nitrilotriacetic acid chromatography (F.C. Lanfermeijer and M.G. Palmgren, unpublished data). This protein was used as a standard for immunoquantification.

Immunolocalization of Expressed Plant H^+-ATPase in Yeast Cells

Yeast cells were grown on galactose, and detection of plant H^+-ATPase by immunolocalization was performed as described by Pringle et al. (1991). The yeast cells were fixed with 3.5% formaldehyde and washed; spheroplasts, obtained by digestion with zymolase, were labeled with monoclonal antibody 46E5/B11C10 raised against the plasma membrane H^+-ATPase (Villalba et al., 1992).

ACKNOWLEDGMENTS

This work was funded by the Danish Natural Science Research Council, NOVO Nordisk Fonden, and the European Communities' BIOTECH Programme as part of the Project of Technological Priority 1993–1996. F.C.L. is a fellow of the Danish Programme for Visiting Scientists financed by the Danish Research Academy.

Received March 31, 1995; accepted July 26, 1995.

REFERENCES

Anderson, J.A., Huprikar, S.S., Kochian, L.V., Lucas, W.J., and Gaber, R.F. (1992). Functional expression of a probable *Arabidopsis thaliana* potassium channel in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **89,** 3736–3740.

Baginsky, E.S., Foa, P.P., and Zak, B. (1967). Determination of phosphate: Study of labile organic phosphate interference. Clin. Chim. Acta **15,** 155–158.

Blatt, M.R. (1988). Mechanisms of fusicoccin action: A dominant role for secondary transport in a higher-plant cell. Planta **174,** 187–200.

Bradford, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. **72,** 248–254.

Brauer, D., and Tu, S.-I. (1995). Effects of nucleotide analogs on ATP hydrolysis by P-type ATPases: Comparison between the canine kidney ($Na^+ + K^+$) ATPase and maize root H^+-ATPase. Physiol. Plant. **93,** 526–532.

Cantley, L.C., Cantley, L.G., and Josephson, L. (1978). A characterization of vanadate interactions with the (Na,K)-ATPase: Mechanistic and regulatory implications. J. Biol. Chem. **253,** 7361–7368.

Cid, A., Perona, R., and Serrano, R. (1987). Replacement of the promoter of the yeast plasma membrane ATPase gene by a galactose-dependent promoter and its physiological consequences. Curr. Genet. **12,** 105–110.

Eraso, P., and Portillo, F. (1994). Molecular mechanism of regulation of yeast plasma membrane H+-ATPase by glucose. J. Biol. Chem. **269,** 10393–10399.

Frommer, W.B., Hummel, S., and Riesmeier, J.W. (1993). Expression cloning in yeast of a cDNA encoding a broad specificity amino acid permease from *Arabidopsis thaliana.* Proc. Natl. Acad. Sci. USA **90,** 5944–5948.

Frommer, W.B., Hummel, S., and Rentsch, D. (1994). Cloning of an *Arabidopsis* histidine transporting protein related to nitrate and peptide transporters. FEBS Lett. **347,** 185–189.

Hager, A., Debus, G., Edel, H.-G., Stransky, H., and Serrano, R. (1991). Auxin induces exocytosis and the rapid synthesis of a high turnover pool of plasma-membrane H+-ATPase. Planta **185,** 527–537.

Harper, J.F., Surowy, T.K., and Sussman, M.R. (1989). Molecular cloning and sequence of cDNA encoding the plasma membrane proton pump (H+-ATPase) of *Arabidopsis thaliana.* Proc. Natl. Acad. Sci. USA **86,** 1234–1238.

Harper, J.F., Manney, L., De Witt, N.D., Yoo, M.H., and Sussman, M.R. (1990). The *Arabidopsis thaliana* plasma membrane H+-ATPase multigene family. J. Biol. Chem. **265,** 13601–13608.

Harper, J.F., Manney, L., and Sussman, M.R. (1994). The plasma membrane H+-ATPase family in *Arabidopsis*: Genomic sequence of *AHA10* which is expressed primarily in developing seeds. Mol. Gen. Genet. **243,** 572–587.

Hill, J.E., Myers, A.M., Koerner, T.J., and Tzagoloff, A. (1986). Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. Yeast **2,** 163–167.

Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K., and Pease, L.R. (1989). Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77,** 51–59.

Houlné, G., and Boutry, M. (1994). Identification of an *Arabidopsis thaliana* gene encoding a plasma membrane ATPase whose expression is restricted to anther tissues. Plant J. **5,** 311–317.

Hsu, L.-C., Chiou, T.-J., Chen, L., and Bush, D.R. (1993). Cloning a plant amino acid transporter by functional complementation of a yeast amino acid transport mutant. Proc. Natl. Acad. Sci. USA **90,** 7441–7445.

Ito, H., Fukuda, K., and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153,** 163–168.

Johansson, F., Sommarin, M., and Larsson, C. (1993). Fusicoccin activates the plasma membrane H+-ATPase by a mechanism involving the C-terminal inhibitory domain. Plant Cell **5,** 321–327.

Kwart, M., Hirner, B., Hummel, S., and Frommer, W.B. (1993). Differential expression of two related amino acid transporters with differing substrate specificity in *Arabidopsis thaliana.* Plant J. **4,** 993–1002.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227,** 680–685.

Lanfermeijer, F.C., and Prins, H.B.A. (1994). Modulation of H+-ATPase activity by fusicoccin in plasma membrane vesicles from oat (*Avena sativa* L.) roots. Plant Physiol. **104,** 1277–1285.

Marrè, E. (1979). Fusicoccin: A tool in plant physiology. Annu. Rev. Plant Physiol. **30,** 273–288.

Monk, B.C., Montesinos, C., Leonard, K., and Serrano, R. (1989). Sidedness of yeast plasma membrane vesicles and mechanisms of activation of the ATPase by detergents. Biochim. Biophys. Acta **981,** 226–234.

Ninnemann, O., Jauniaux, J.-C., and Frommer, W.B. (1994). Identification of a high affinity NH_4^+ transporter from plants. EMBO J. **13,** 3464–3471.

Nothwehr, S.F., and Stevens, T.H. (1994). Sorting of membrane proteins in the yeast secretory pathway. J. Biol. Chem. **269,** 10185–10188.

Palmgren, M.G. (1991). Regulation of plant plasma membrane H+-ATPase activity. Physiol. Plant. **83,** 314–323.

Palmgren, M.G., and Christensen, G. (1993). Complementation in situ of the yeast plasma membrane H+-ATPase gene *pma1* by a H+-ATPase gene from a heterologous species. FEBS Lett. **317,** 216–222.

Palmgren, M.G., and Christensen, G. (1994). Functional comparisons between plant plasma membrane H+-ATPase isoforms expressed in yeast. J. Biol. Chem. **269,** 3027–3033.

Palmgren, M.G., Sommarin, M., Ulvskov, P., and Jørgensen, P.L. (1988). Modulation of plant plasma membrane H+-ATPase from oat roots by lysophosphatidylcholine, free fatty acids and phospholipase A_2. Physiol. Plant. **74,** 11–19.

Palmgren, M.G., Sommarin, M., Serrano, R., and Larsson, C. (1991). Identification of an autoinhibitory domain in the C-terminal region of the plant plasma membrane H+-ATPase. J. Biol. Chem. **266,** 20470–20475.

Pardo, J.M., and Serrano, R. (1989a). Structure of a plasma membrane H+-ATPase gene from the plant *Arabidopsis thaliana.* J. Biol. Chem. **264,** 8557–8562.

Pardo, J.M., and Serrano, R. (1989b). Structure of a plasma membrane H+-ATPase gene from *Arabidopsis thaliana* and amino acid sequence of three isoforms. In Plant Membrane Transport: The Current Position, J. Dainty, M.I. de Michelis, E. Marrè, and F. Rasi-Caldogno, eds (Amsterdam: Elsevier), pp. 499–500.

Perez, C., and Boutry, M. (1994). The multigene family of the H+-ATPase of plant plasma membranes. In Membrane Transport in Plants and Fungi: Molecular Mechanisms and Control, M.R. Blatt, R.A. Leigh, and D. Sanders, eds (Cambridge: Company of Biologists Limited), pp. 11–31.

Pringle, J.R., Adams, A.E.M., Drubin, D.G., and Haarer, B.K. (1991). Immunofluorescence methods for yeast. Methods Enzymol. **194,** 565–602.

Rasi-Caldogno, F., Pugliarello, M.C., Olivari, C., and De Michelis, M.I. (1993). Controlled proteolysis mimics the effect of fusicoccin on the plasma membrane H+-ATPase. Plant Physiol. **103,** 391–398.

Riesmeier, J.W., Willmitzer, L., and Frommer, W.B. (1992). Isolation and characterization of a sucrose carrier cDNA from spinach by functional expression in yeast. EMBO J. **11,** 4705–4713.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74,** 5463–5467.

Sauer, N., and Stolz, J. (1994). SUC1 and SUC2: Two sucrose transporters from *Arabidopsis thaliana*: Expression and characterization in baker's yeast and identification of the histidine-tagged protein. Plant J. **6,** 67–77.

Sauer, N., Friedländer, K., and Gräml-Wicke, U. (1990). Primary structure, genomic organization and heterologous expression of a glucose transporter from *Arabidopsis thaliana.* EMBO J. **9,** 3045–3050.

Schachtman, D.P., and Schroeder, J.I. (1994). Structure and transport mechanism of a high-affinity potassium uptake transporter from higher plants. Nature **370,** 655–658.

Sentenac, H., Bonneaud, N., Minet, M., Lacroute, F., Salmon, J.-M., Gaymard, F., and Grignon, C. (1992). Cloning and expression in yeast of a plant potassium ion transport system. Science **256,** 663–665.

Serrano, R., Portillo, F., Monk, B.C., and Palmgren, M.G. (1992). The regulatory domain of fungal and plant plasma membrane H^+-ATPase. Acta Phys. Scand. **146,** 131–136.

Smith, F.W., Ealing, P.M., Hawkesford, M.J., and Clarkson, D.T. (1995). Plant members of a family of sulfate transporters reveal functional subtypes. Proc. Natl. Acad. Sci. USA **92,** in press.

Steiner, H.-Y., Song, W., Zhang, L., Naider, F., Becker, J.M., and Stacey, G. (1994). An Arabidopsis peptide transporter is a member of a new class of membrane transport proteins. Plant Cell **6,** 1289–1299.

Supply, P., Wach, A., Thinès-Sempoux, D., and Goffeau, A. (1993). Proliferation of intracellular structures upon overexpression of the PMA2 ATPase in *Saccharomyces cerevisiae.* J. Biol. Chem. **268,** 19744–19752.

Villalba, J.M., Palmgren, M.G., Berberian, G.E., Ferguson, C., and Serrano, R. (1992). Functional expression of plant plasma membrane H^+-ATPase in yeast endoplasmic reticulum. J. Biol. Chem. **267,** 12341–12349.

Wright, R., Basson, M., D'Ari, L., and Rine, J. (1988). Increased amounts of HMG-CoA reductase induce "karmellae": A proliferation of stacked membrane pairs surrounding the yeast nucleus. J. Cell Biol. **107,** 101–114.